Chapter 6

Designing Novel Breeding Strategies for Producing High-Oil Crops Based on a Molecular Understanding of Triacylglycerol Metabolism

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Abstract

Seeds are storage organ in plants and main resource of plant oils to human civilization and the demand of plant oils are increasing yearly and expansion of the production capacity is an urgent issue worldwide. Thus, it is necessary to improve oil yields per unit area and generation of crops with high-oil content is needed. *Arabidopsis thaliana* plays a vital role in advancement of genetics and molecular biology in plant sciences. The forward and reverse genetic approaches with Arabidopsis have provided an overview of triacylglycerol metabolism. The elucidation of the overview contributes to understanding of spatiotemporal regulation of a metabolic flow of triacylglycerol metabolism in plant cell. This understanding sheds light on bottlenecks in triacylglycerol biosynthesis and provides novel clues for increasing seed triacylglycerol content. Recent advance in metabolic engineering approaches demonstrate several evidences that triacylglycerol metabolism is coordinated with other metabolisms. Most notably, triacylglycerol biosynthesis competes with biosynthesis of starch or seed storage proteins. These studies indicate that alterations of the metabolic pathways to avoid the competitions could be a novel concept for increasing seed oil content.

Keywords: Seed, oil, Triacylglycerol, Metabolic engineering, *Arabidopsis thaliana*

1. Introduction

Seeds are storage organs in plants that accumulate massive quantities of carbohydrates, proteins, and oils, which are collectively referred to as seed storage reserves. Seed storage reserves are

utilized to supply energy and nutrients to the embryo during postgerminative growth until the plant acquires the ability to perform photosynthesis. Hence, seed storage reserves are an easily degradable source of organic matter for organisms.

Seeds are widely used as industrial materials. For example, plant oils are mainly derived from seeds. In 2014, the overall consumption of plant oils was 170,980 kt, which is nearly double that consumed in 2004. This increase is attributed to a greater demand for dietary oil, as well as industrial materials for use as carbon-neutral oils, representing an alternative to petroleum.

To improve oil productivity in plants, it is essential to increase seed yields in crops. However, the agricultural area worldwide has been flat for 40 years. Therefore, improving seed yield per plant has become increasingly important. Since increasing seed yield is one of the major issues in plant science, effective strategies for increasing yield have been investigated. Considering the many successes in the field of metabolic engineering of microorganisms, metabolic engineering represents a promising approach for increasing oil yields in seeds. Lipid metabolism has been extensively studied, and its metabolic pathways and regulatory systems have been elucidated. Additionally, in-depth analysis of crop genomes has been greatly expedited by recent advances in life science technologies (next-generation sequencing technology, genome editing, and so on). Therefore, numerous translational studies of model plant species have been performed, which have shed light on crop species. This chapter summarizes recent advances in our understanding of oil metabolism in seeds and introduces promising strategies for increasing oil production in crops.

2. Main components of seeds

Seeds accumulate carbohydrates, proteins, and oils as seed storage reserves. The ratios of these components dramatically differ among plant species, representing a major factor that determines the usage and application of seeds [1].

2.1. Cereal seeds

Starch consists of carbohydrates, which comprise polysaccharides composed of glucose [2–4]. Major cereal seeds, such as rice, wheat, and maize, contain large amounts of starch. Barley and rye seeds also accumulate starch as their main storage reserves. The starch contents in these cereal seeds are over 70%, whereas the contents of proteins and oils are less than 20% (**Table 1**). Therefore, cereal seeds represent an important energy source for animals.

2.2. Oil seeds

Oil seeds mainly accumulate lipids. Oil seeds are one of the major sources of plant oils. These seeds are compressed and heated to extract oils from their cells. Oil crops, which produce oil seeds, are cultivated worldwide. Oil palm, soybean, and rapeseed, the "big three", represent three-quarters of total plant oil production (**Table 2**). Palm oil and olive oil are extracted from fruits, whereas many other oils are extracted from seeds (**Table 2**).

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Grain	Carbohydrates	Proteins	Oils
wheat	72.2	10.6	3.1
rice	73.8	6.8	2.7
maize	70.6	8.6	5.0
barley	72.1	10.9	2.1
rye	70.7	12.7	2.7

Table 1. Carbohydrate, protein, and fat contents in cereal seeds. Values (%/w) in this table were extracted from the Food Composition Database of the Ministry of Education, Culture, Sports, Science, and Technology in Japan (http://fooddb.mext.go.jp/index.pl).

	Plant species	Production (kilotons, 2013–2014)	Rate (% total plant oil production)	Storage organ
1	oil palm	59,360	34.09	fruit
2	soybean	44,439	25.52	seed
3	rapeseed	27,029	15.52	seed
4	sunflower	16,169	9.29	seed
5	cotton	4,859	2.79	seed
6	peanut	3,548	2.04	seed
7	olive	3,416	1.96	fruit
8	corn	3,148	1.80	seed

Table 2. Major oil crops and worldwide oil production in 2013–2014. The rates of total production and % total plant oil production are summarized for major oil crops; the values were obtained from FAOSTAT (http://faostat.fao.org/).

Species	Concentration (%)		Variety
	Oil	Protein	
soybean	20.1	41.4	Kariyutaka
rapeseed	41.0	25.6	Kizakinonatane
sunflower	33.8	30.4	Mammoth Russian
peanut	42.6	25.8	Omasari
sesame	49.9	21.6	–

Table 3. Oil and protein concentrations in major oil seed crops. The oil and protein concentrations were measured following the methods of Kanai et al. (2016). The variety of sesame used in this experiment was not identified.

2.2.1. Oil contents of oil seeds

Of the major oil crops, the oil content of oil palm fruit is over 50%, whereas that of soybean and rapeseed is 20.1% and 41%, respectively (**Table 3**) [5, 6]. The seed oil content in sunflower, peanut, and sesame is 33.8%, 42.6%, and 49.9%, respectively (**Table 3**). Thus, the seed oil contents of typical oil crops range from approximately 20–50%. These values are relatively low compared to the starch contents of rice, wheat, maize, and so on, suggesting that there is strong potential for increasing oil contents in seeds.

2.2.2. Protein contents of oil seeds

The protein content of major cereal seeds is less than 15% (**Table 1**), whereas the protein content of soybean and rapeseed, which are major suppliers of plant oils, is 41.4% and 25.6%, respectively (**Table 3**). Most oil seeds accumulate more proteins, i.e., seed storage proteins (hereafter referred to as SSPs), than cereal seeds.

3. Triacylglycerol metabolism in seeds

Lipids are essential components not only for animals, but also for plants. Various species of lipids are biosynthesized throughout plant organs. Some lipids, such as membrane lipids [7], cuticular waxes [8], and volatile oils, comprising fatty acids [9–11], alcohols, terpenes, and so on, are produced in plants in response to changes in the external environment. On the other hand, seed oils, the primary subject of this chapter, are composed of triacylglycerol (referred to hereafter as TAG) [12–14]. Except for palm oil, major seed oils are liquids at room temperature (**Figure 1A**). One molecule of TAG is composed of a glycerol backbone and three fatty acids (**Figure 1B**).

3.1. Triacylglycerol biosynthesis

TAG biosynthesis primarily involves two steps, i.e., fatty acid biosynthesis and the assembly of fatty acids with a glycerol backbone (**Figure 1C**).

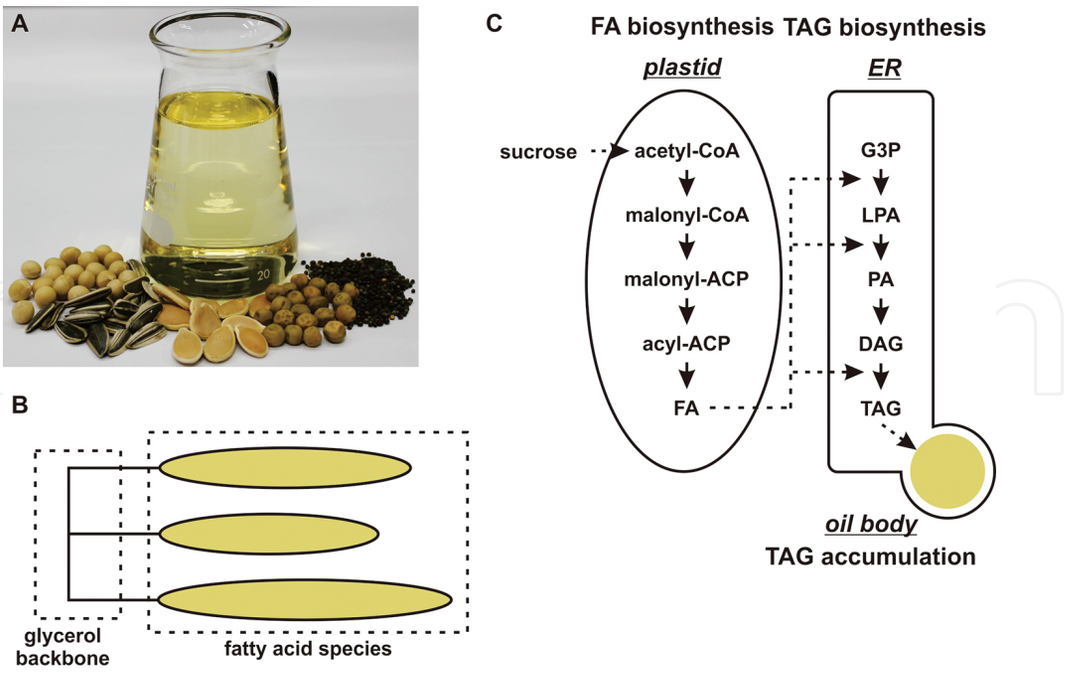

Figure 1. Triacylglycerol in plant seeds. (**A**) Plant oil extracted from seeds. (**B**) Structure of TAG. TAG is produced by the assembly of one glycerol with three fatty acids. (**C**) Schematic diagram of TAG biosynthesis in plant cells.

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3.2. Fatty acid biosynthesis

In plant cells, fatty acids are synthesized in plastids. Fatty acid biosynthesis begins with the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (reaction (1) in **Figure 2**). The malonyl group in malonyl-CoA is transferred to acyl carrier protein (hereafter referred to as ACP), producing malonyl-ACP (reaction (2) in **Figure 2**). Next, 3-keto-butyryl-ACP is synthesized via the condensation reaction of malonyl-ACP with acetyl-CoA (reaction (3) in **Figure 2**). The 3-keto-butyryl-ACP molecule is converted to butyryl-ACP via reduction and dehydration (reaction (4) in **Figure 2**). The provision of C2 units, reduction, and dehydration are repeated, leading to the elongation of the carbon chain of acyl-ACP (carbon chain elongation in **Figure 2**). Synthesized acyl-ACP is catalyzed by thioesterase to form free fatty acids, which are again converted to acyl-CoA and imported to the endoplasmic reticulum (hereafter referred to as ER).

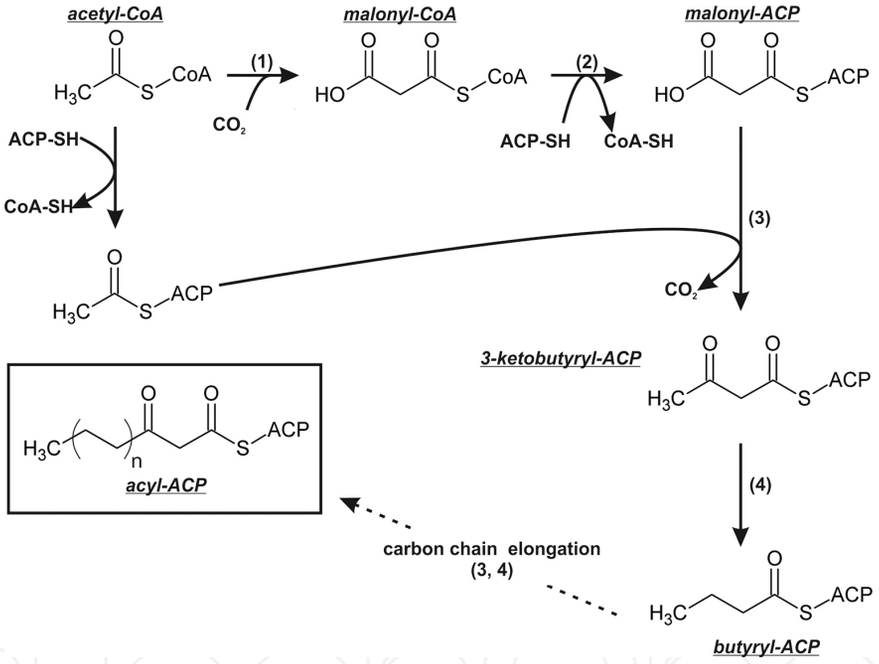

Figure 2. Fatty acid biosynthesis in plastids: (1) acetyl-CoA carboxylase, (2) malonyl CoA:ACP transacylase, (3) 3-ketoacyl-ACP synthase, and (4) 3-ketoacyl-ACP reductase, 3-hydroxyacyl ACP dehydrase, and enoyl-ACP reductase.

3.3. Assembly of fatty acids with the glycerol backbone

Acyl-CoA is a donor of the acyl-group for TAG biosynthesis. *De novo* synthesis of TAG begins with the transfer of the acyl-group to the sn-1 position of glycerol 3-phosphate, leading to the production of lysophosphatidic acid (reaction (1) in **Figure 3**). Subsequently, phosphatidic acid is produced by the transfer of the acyl group to the sn-2 position (reaction (2) in **Figure 3**). Next, the sn-3 position of phosphatidic acid is dephosphorylated and converted to diacylglycerol (reaction (3) in **Figure 3**), and, finally, TAG is synthesized by the transfer of the acyl-group to the sn-3 position of diacylglycerol (hereafter referred to as DAG) (reaction (4) in **Figure 3**). In

addition to the *de novo* synthesis pathway, TAG is produced via an alternative pathway through phosphatidylcholine (hereafter referred to as PC) [15, 16]. PC, one of the main components of membrane lipids, is present in pools in the ER [16]. PC pools affect *de novo* TAG synthesis and the acyl-CoA pool in the cytosol to supply DAG as a precursor for TAG [16].

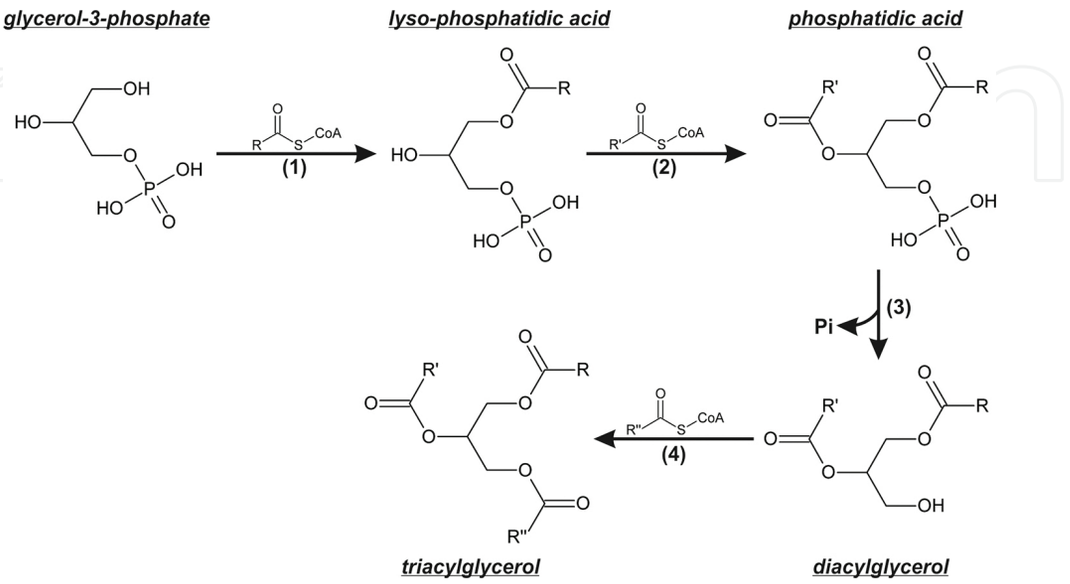

Figure 3. Triacylglycerol formation in the ER: (1) acyl-CoA:G3P acyltransferase, (2) acyl-CoA:LPA acyltransferase, (3) PA phosphatase, and (4) acyl-CoA:DAG acyltransferase.

3.4. Triacylglycerol accumulation

Synthesized TAGs accumulate in compartments in the ER (**Figure 1C**), which are converted to vesicles through budding. The vesicles then develop into oil-accumulating organelles, i.e., oil bodies [16–19]. Oil bodies are TAG storage organelles with a single layer membrane, whose major membrane protein is oleosin [18, 20, 21]. Oleosin blocks adhesion between neighboring oil bodies, which allows small oil bodies to be packed tightly together without adhesion in the cells of oil seeds [22].

4. Strategies for improving oil content by modifying triacylglycerol metabolism in oil seed crops

Since plant oils are commercially important, improving oil seed crops has long been a focus of breeders. Such breeding efforts, which began in ancient times, have led to the improvement of oil contents in several crops. In addition, the oil contents of the seeds of modern cultivars are significantly higher than those of wild species [23, 24]. Currently, many plant breeders have undertaken the challenge of further increasing seed oil contents. However, recent studies using quantitative trait loci analyses revealed that seed oil contents are controlled by many

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genes with additive effects [25–28], suggesting that traditional breeding methods based on cross-fertilization may be inadequate for further increasing seed oil contents.

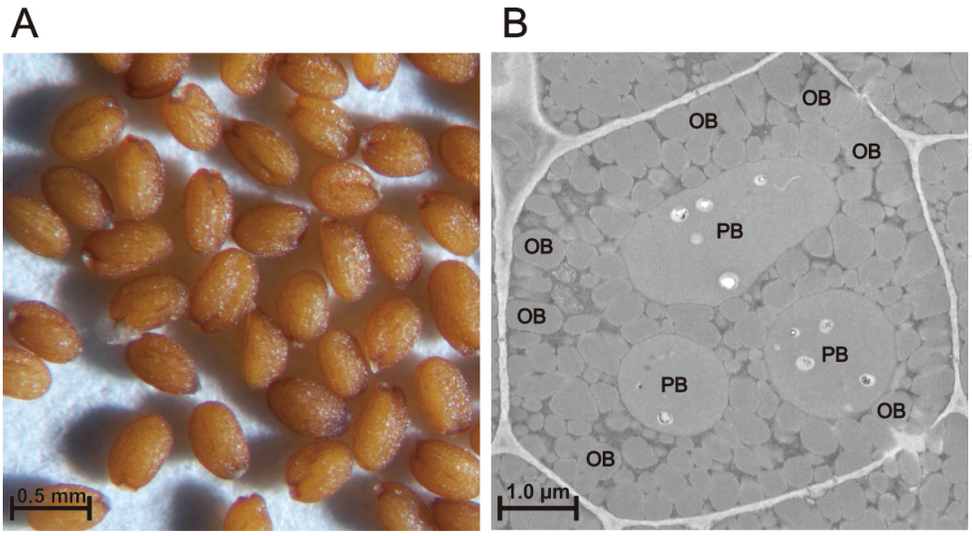

Figure 4. Seeds of *Arabidopsis thaliana*. (A) Microscopic image of Arabidopsis seeds. (B) Electron microscopic image of Arabidopsis seed cells. OB: oil body, an oil storage organelle in seeds. PB: protein body, an organelle that accumulates seed storage protein.

By contrast, genetic transformation may be an effective tool for increasing seed oil contents. The model plant *Arabidopsis thaliana*, a close relative of the major oil crop *Brassica napus*, produces typical oil seeds (**Figure 4A** and **B**). The use of Arabidopsis drastically simplifies both the processes of screening mutants with abnormal phenotypes and generating transformants, in which genes of interest are introduced. The use of Arabidopsis has accelerated the process of uncovering TAG metabolic pathways in plants. In fact, most genes encoding enzymes involved in TAG metabolism have been identified based on characterization of Arabidopsis mutants defective in TAG metabolism [29, 30]. Elucidating TAG metabolic pathways has revealed the limiting factors and key enzymes in this process and has led to the development of novel strategies for improving seed TAG contents. In this section, we review important strategies for improving TAG contents in seeds based on metabolic engineering approaches.

4.1. Enhancement of TAG biosynthesis

The TAG biosynthetic pathway, which extends across plastids, the cytosol, and the ER, involves many enzymes [30]. Additionally, DAG biosynthetic pathways include the *de novo* synthetic pathway and the phosphatidylcholine-derived pathway (hereafter referred to as PC-derived pathway), whose activities greatly differ among plant species [15, 16]. Thus, increasing the activity of enzymes in the *de novo* or PC-derived pathway does not always increase seed oil contents in every plant species. On the other hand, TAG production from DAG by acyl-CoA:DAG acyltransferase is a common pathway among plants. Overexpressing acyl-CoA:DAG acyltransferase significantly increases seed TAG contents in Arabidopsis [31] and in other plants species [32–37]. These findings indicate that the conversion of DAG to TAG is

one of the rate-limiting steps in the production of TAG in seeds, and they suggest that the acyl-CoA:DAG acyltransferase gene would be a promising target gene for increasing TAG contents.

4.2. Suppression of TAG degradation

Synthesized TAG is stored in oil bodies and degraded during germinative growth [17, 38, 39]. The TAG degradation pathway has also been uncovered, and most genes encoding enzymes in this pathway have been identified [17, 39]. The expression of these genes is upregulated after seed imbibition, and TAG degradation activity rapidly increases in imbibed seeds [40–42]. These genes are also expressed during seed development in several plants [43, 44]. In fact, TAG degradation occurs in developing seeds [45–47]. Therefore, the TAG degradation pathway is activated during seed development, and seeds lose some of the TAG synthesized during seed development. This finding suggests that suppressing TAG degradation would be a promising strategy for improving seed oil contents. Oil degradation begins with TAG hydrolysis via TAG lipase. TAG lipase was genetically identified as *SUGAR DEPENDENT 1* in Arabidopsis [48] and was subsequently identified in rapeseed and Jatropha [47, 49]. Suppressing *SUGAR DEPENDENT 1* expression significantly increases seed oil contents [47, 49]. These reports indicate that suppressing TAG degradation via suppressing *SUGAR DEPENDENT 1* expression may represent an effective strategy for increasing seed oil contents in oil seed crops.

5. Novel strategies focused on carbon flux in seeds

In production systems that utilize microorganisms and cultured cells for metabolic engineering, individual cells synthesize organic compounds, providing a limitless source of carbon in the medium. By contrast, the seed, representing only one of many plant organs, synthesizes storage reserves using a limited carbon source provided by photosynthesis. This limited carbon source is thought to represent another limiting factor to the use of seeds as production systems, in addition to the limited activities of metabolic enzymes. To utilize seeds for oil production, it is important to obtain a comprehensive understanding of the factors that regulate TAG production, including carbon flow, metabolite transport, TAG metabolism, compartmentation, competition between other reserves, and so on. Recent studies identifying other factors that limit oil production in seeds have opened up the possibility of developing novel strategies for improving seed oil contents.

5.1. Effective utilization of “the window” during seed development

Fertilized embryos grow into mature seeds through sequential development. Seed storage oils are produced only during a short period of seed development; high TAG biosynthesis activity only occurs for a short period of time. The master transcription factor of TAG biosynthesis, WRINKLED1 (hereafter referred to as WRI1), is expressed during this short period and induces the expression of genes related to fatty acid biosynthesis [50–54]. Although WRI1 positively regulates TAG biosynthesis, the overexpression of *WRI1* fails to increase TAG contents in seeds

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[51, 53]. These findings suggest that, in order to increase seed TAG contents, it is necessary to reconsider the timing and duration of *WRI1* expression. Arabidopsis seeds initially accumulate TAG, followed by proteins [1]. A detailed analysis of seed development revealed that there is a time lag between the termination of TAG biosynthesis and the initiation of protein biosynthesis, i.e., there is a "window" period in the middle phase of seed development during which the activities of the TAG and protein synthesis pathways are low (**Figure 5**) [54]. Additionally, strong expression of *WRI1* during this window extends the duration of TAG biosynthesis and increases TAG contents in seeds [54], indicating that *WRI1* expression during this window effectively induces the TAG biosynthesis pathway. This finding has important implications for developing novel strategies for increasing seed TAG contents. The timing and duration of TAG and seed storage protein synthesis differ greatly among plant species. Therefore, identifying the window phase and fine tuning of *WRI1* expression are essential for generating oil crops with increased TAG contents in seeds.

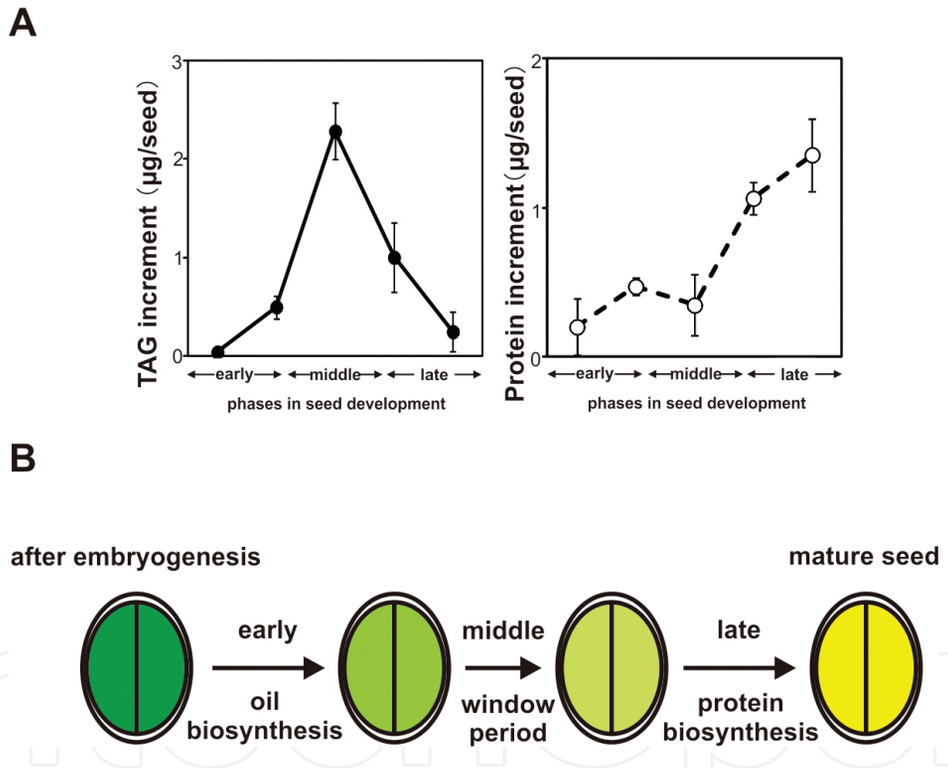

Figure 5. Oil and protein biosynthesis during seed development. (A) Phases of oil and protein biosynthesis during seed development in *Arabidopsis thaliana*. (B) Schematic diagram of oil and protein biosynthesis during seed development in *Arabidopsis thaliana*.

5.2. Concentrating the carbon source into triacylglycerol biosynthesis by reducing the levels of other organic materials

Seeds accumulate various organic materials, including carbohydrates and proteins, in addition to TAG. Therefore, reducing carbohydrate and protein levels would help direct the carbon source into TAG biosynthesis.

5.2.1. Reducing polysaccharide biosynthesis

Seeds are generally covered with seed coats. Members of the Brassica family, including Arabidopsis, rapeseed, and so on, accumulate large amounts of mucilage consisting of pectin [55, 56]. Therefore, a considerable volume of the carbon source is consumed by mucilage production. Shen et al. reported that knockout of *GLABRA2*, which encodes a WRKY transcription factor controlling epidermal development, increases seed oil content in Arabidopsis [57]. Subsequent research demonstrated that increasing oil contents by suppressing *GLABRA2* expression reduces mucilage biosynthesis [58]. These findings indicate that reducing mucilage biosynthesis causes the carbon source to be directed into TAG biosynthesis. This transfer of the carbon source via reduction of polysaccharide levels has been verified in leaves. Starch comprises one of the main storage reserves in leaves. On the other hand, TAG biosynthesis activity is quite low in leaves. Therefore, to accumulate TAG in leaves, it is essential to both suppress starch biosynthesis and activate TAG biosynthesis. Sanjaya et al. found that overexpressing *WRI1* while suppressing the production of a small subunit of adenosine diphospho (ADP)-glucose pyrophosphorylase, a key enzyme for starch biosynthesis, significantly increases TAG contents in leaves [59]. These results indicate that reducing polysaccharide biosynthesis leads to the funneling of the carbon source into TAG biosynthesis, representing a possible novel strategy for increasing seed TAG contents in oil crops.

5.2.2. Reducing seed storage protein biosynthesis

As described above, oil seeds accumulate large amounts of seed storage proteins in addition to TAG. Crop breeders have reported a negative correlation between TAG and protein contents, especially in soybean [60, 61], suggesting that reducing the levels of seed storage proteins increases TAG contents in seeds. However, in Arabidopsis, knockout of genes encoding major seed storage proteins has little effect on TAG contents in seeds, although the protein content is reduced [54, 62]. These results may be due to the time lag between TAG and protein biosynthesis (**Figure 5**): little of the surplus carbon source derived from the suppression of protein biosynthesis is utilized for TAG biosynthesis because the TAG biosynthesis activity is quite low when protein synthesis is active in the late phase of seed development [54]. Therefore, simultaneously overexpressing *WRI1* and reducing protein synthesis greatly increases seed TAG contents due to effective utilization of the surplus carbon source [54]. This finding indicates that reducing protein synthesis indeed provides the surplus carbon source required for TAG production which, when combined with the simultaneous activation of TAG biosynthesis, leads to increased TAG production. This finding also demonstrates that the combination of these functional strategies has an additive effect on seed TAG content (**Figure 6**) [54, 63]. Breeding lines with reduced SSP content have been established in several crops [64, 65]. Introduction of *WRI1* into these breeding lines represents a potentially important strategy for high-oil seed production.

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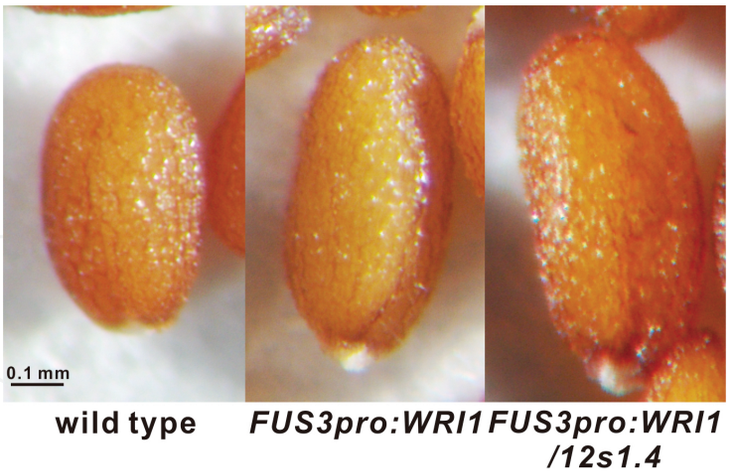

Figure 6. During the enlargement of seeds, the activation of TAG biosynthesis and the reduction of protein synthesis occur simultaneously. *FUS3pro:WRI1*; seeds from transgenic plants harboring *WRI1* under the control of the *FUS3* promoter in wild-type plants (Col-0): *WRI1* is expressed during the window phase. *FUS3pro:WRI1/12s1.4*; seeds from transgenic plants harboring *WRI1* under the control of the *FUS3* promoter in a double knockout mutant of *12S1* and *12S4*, encoding major seed storage proteins in *Arabidopsis thaliana*.

6. Conclusion

Molecular genetic analysis of Arabidopsis has led to the incredibly rapid elucidation of the mechanisms underlying the metabolism and regulation of seed storage reserves. The results from these basic studies provide powerful clues to help solve important issues in crop breeding. Optimizing the strategies developed based on the results of basic studies for use in crops will lead to crucial innovations for improving crop yields.

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